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Functional and Biological Properties of an Avian Variant Long Terminal Repeat Containing Multiple A to G Conversions in the U3 Sequence

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We previously reported that infection of chicken embryonic neuroretina cells with Rous-associated virus type 1 leads to the frequent occurrence of spliced readthrough transcripts containing viral and cellular sequences. Generation of such chimeric transcripts constitutes a very early step in oncogene transduction. We report, here, the isolation of a c-*mil* transducing retrovirus, designated IC4, which contains a highly mutated U3 sequence in which 48% of A is converted to G. Functional analysis of this variant U3 indicated that these mutations do not impair viral transcription and replication; however, they abolish functioning of its polyadenylation signal, thus allowing readthrough transcription of downstream cellular sequences. On the basis of these results, we designed a nonreplicative retroviral vector, pIC4Neo, expressing the neomycin resistance (Neo^r) gene under the control of the IC4 long terminal repeat. Infection of nondividing neuroretina cells with virus produced by a packaging cell line transfected with pIC4Neo occasionally resulted in sustained cell proliferation. Two independent G418-resistant proliferating cultures were found to express hybrid RNAs containing viral and cellular sequences. These sequences were characterized by reverse transcription-PCR and were identified in both cultures, suggesting that proliferation was correlated with a common integration locus. These results indicate that IC4Neo virus functions as a useful insertional mutagen and may allow identification of genes potentially involved in regulation of cell division.

We previously reported that infection of cultured chicken embryonic neuroretina (NR) cells with Rous-associated virus type 1 (RAV-1), which does not carry an oncogene, results in reproducible transduction of two related serine/threonine protein kinases, c-*mil*/c-*raf* and c-R*mil*/B-*raf* (11, 27, 28). These newly generated viruses were selected by their ability to induce proliferation of postmitotic NR cells. Analysis of successive steps resulting in the emergence of oncogene-containing retroviruses led us to propose a general mechanism for retroviral activation and transduction of proto-oncogenes. According to this model, the very early events leading to the constitution of 5′ junctions between viral and cellular sequences take place at the RNA level (10–12). These events presumably involve the synthesis of readthrough transcripts containing viral and downstream cellular sequences from a RAV-1 provirus integrated upstream of proto-oncogenes. Such transcripts, initiated at the 5′ long terminal repeat (LTR) of the RAV-1 provirus, would occasionally escape cleavage and polyadenylation monitored by the polyadenylation signal (PAS) of the 3′ LTR (19, 44, 46). Primary readthrough transcripts could then undergo alternative splicing between the viral leader and activated proto-oncogene exons, as previously reported (10–12). Alternatively spliced chimeric transcripts, which result in activation of cellular sequences, have also been described for other systems, with both avian (29) and murine (35) retroviruses. We report here the isolation of a c-*mil*/c-*raf* transducing retrovirus, designated IC4, carrying a highly mutated U3 sequence, in which 48% of A is converted to G. We show that, as a consequence of these mutations, the PAS in the IC4 LTR is not functional, whereas viral transcription and replication are not impaired. This suggests that viruses carrying this variant LTR could be instrumental in retroviral activation of cellular genes by increasing the frequency at which readthrough transcripts are generated. Consequently, we designed a replication-defective retroviral vector, pIC4Neo, containing the neomycin resistance (Neo^r) gene under the control of the mutated IC4 LTR, and we used it as an insertional mutagen to activate NR cell division. We show that infection with IC4Neo virus occasionally results in long-term proliferation of G418-resistant NR cells. As expected, proliferating NR cells synthesize abundant readthrough transcripts containing cellular sequences. Characterization of such chimeric transcripts should help to identify sequences downstream of the viral integration site and to assess their roles in regulation of NR cell division.

MATERIALS AND METHODS

Cell culture and viruses. NR cultures were prepared from 8-day-old Brown Leghorn chicken embryos (Gs$^+$ Chf$^+$) of the Edinburgh strain, as previously described (33). Cultures were maintained and passaged in Eagle basal medium supplemented with 5 to 10% fetal calf serum.

Chicken embryo fibroblasts (CEF) were prepared from 11-day-old embryos by standard procedures and grown in Dulbecco's modified Eagle medium containing 5% newborn calf serum and 10% tryptose phosphate broth.

The avian leukosis virus-based quail packaging cell line Isolde, which produces after plasmid transfection a replication-defective virus of subgroup A, was maintained in culture as previously described (7). RAV-1/OR is a subgroup A lymphomatosis virus. Fibroblasts were infected with serial 10-fold dilutions of RAV-1 virus, and the titer of the virus was

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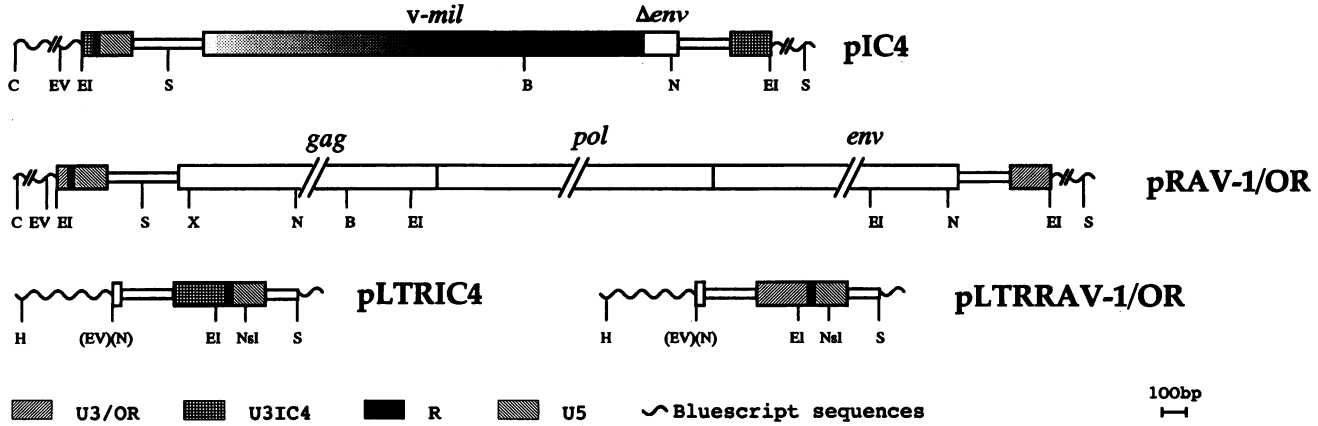

FIG. 1. Genetic organization and restriction mapping of IC4- and RAV-1/OR-derived plasmids. pIC4 was obtained by inserting the *Eco*RI fragment of IC4 provirus into the Bluescript vector (Stratagene). pRAV-1/OR was obtained by inserting the 7.8-kb *Eco*RI fragment of RAV-1/OR provirus into Bluescript. pLTRIC4 and pLTRRAV-1/OR contain the LTRs of IC4 and RAV-1/OR, respectively, inserted into the Bluescript plasmid. These LTRs are flanked by 3′ and 5′ untranslated sequences of RAV-1 (thin white boxes). Restriction enzyme abbreviations: B, *Bgl*II; C, *Cla*I; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nru*I; NsI, *Nsi*I; S, *Sac*I; X, *Xho*I.

determined by resistance to superinfecting, transforming Rous sarcoma virus (RSV) of the same subgroup.

DNA purification and restriction enzyme analysis. High-molecular-weight DNA was purified by standard procedures (17). Plasmid DNAs were purified on Qiagen columns. DNAs were digested with restriction enzymes under conditions recommended by the suppliers (New England Biolabs and Appligene) and analyzed by Southern blotting (42). Hybridization was performed under stringent conditions, as previously described (49), with probes radioactively labeled by nick translation (34).

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was isolated by the guanidium thiocyanate-cesium chloride method (6). RNAs were denatured at 60°C in a formamide-formaldehyde mixture (37), fractionated by electrophoresis in a 1% agarose–2.2 M formaldehyde gel (24), transferred to a nitrocellulose filter in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and hybridized to radioactively labeled probes under stringent conditions.

Molecular cloning and DNA sequencing. Molecular cloning of IC4 provirus into *Eco*RI-digested arms of λgt11 vector was performed under conditions previously described (28).

Restriction fragments were inserted into Bluescript and/or pUC18 plasmids, and their nucleotide sequences on both strands were determined by the dideoxy chain termination method (38), using universal or specific primers.

Plasmids. The genomic organization and restriction mapping of plasmids used to construct the IC4- and RAV-1/OR-derived retroviral vectors are presented in Fig. 1.

pIC4Neo vector was obtained by inserting the *Eco*RI fragment of pRAV-1Neo plasmid (51) into the *Eco*RI site of pLTRIC4. pRAV-1Neo contained the *Eco*RI-*Xho*I 5′ fragment of pRAV-1/OR; the *Xba*I-*Nru*I fragment of pXN22 (2), which provided the neomycin resistance gene together with a splice acceptor site; and the *Nru*I-*Eco*RI 3′ fragment of pRAV-1/OR. In this plasmid, the *Xho*I site was replaced by an *Xba*I site following filling and addition of an *Xba*I linker (New England Biolabs).

Transfection of NR cells and CAT assay. NR cells obtained from 8-day-old chicken embryos were seeded at a density of 4 × 10^6 cells per 35-mm-diameter petri dish and maintained in Eagle's basal medium supplemented with 10% fetal calf serum. The cells were transfected by the calcium phosphate coprecipitation technique 15 h later (16). Each plate received 10 μg of test plasmid and 2 μg of pSVβgal (Pharmacia), in a final concentration of 20 μg/ml. Twenty hours after transfection, cells were harvested, rinsed, and lysed. The β-galactosidase activity in the extracts was measured by the method of Herbomel et al. (18). Extracts corresponding to equal levels of β-galactosidase activity were used for the chloramphenicol acetyltransferase (CAT) assay performed by the method of Gorman et al. (14).

Transfection of COS-1 cells. COS-1 cells were transfected by the DEAE-dextran-chloroquine method (4). The cells were plated at a density of 7 × 10^5 cells per 10-cm-diameter petri dish, in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum; 24 h later, cells received 10 μg of pGAL4polyII, pGAL4ΔIC4polyII, or pGAL4ΔRAV-1polyII construct (see Figure 4). Cultures were lysed 48 h after transfection, and cellular RNAs were analyzed by Northern blotting.

Probes. The following probes were used: the 1.1-kbp *Bam*HI-*Hpa*I v-*mil* fragment of the MH2BS molecular clone (27), the 2.45-kbp *gag*-containing *Eco*RI fragment of the pRAV-1/OR clone, the 200-bp *Hin*dIII-*Cla*I GAL4 fragment of the pGAL4polyII (5, 50), and the Neo^r gene-containing *Xba*I-*Nru*I fragment of pIC4Neo (this fragment also contains 210 bp of viral origin [2] identifying endogenous sequences in chicken cells). The IC4 LTR and leader probes were obtained by the PCR technique. To obtain the IC4 LTR probe, 1 ng of pLTRIC4 was used for amplification by PCR with an IC4 U3-specific primer (AATGTAGTCTTGTGCAATAC) and a U5-specific primer (AATGAAGCCTTCTGCTTCAT). For the leader probe, 1 ng of pRAV-1/OR was amplified with 5′ and 3′ leader primers (TGGTGACCCCGACGTGATC and CTTTATGACGGCTTCCAT, respectively). The PCR product was subcloned into the *Sma*I-*Eco*RV sites of Bluescript vector.

Nucleotide sequence accession numbers. The nucleotide sequences of the IC4 provirus, RAV-1/SF LTR, and RAV-1/OR LTR have been given GenBank accession numbers X77628, X77629, and X77630, respectively.

RESULTS AND DISCUSSION

IC4 retrovirus contains a high rate of A to G conversions in the U3 sequence. Serial passaging of RAV-1 on cultured NR

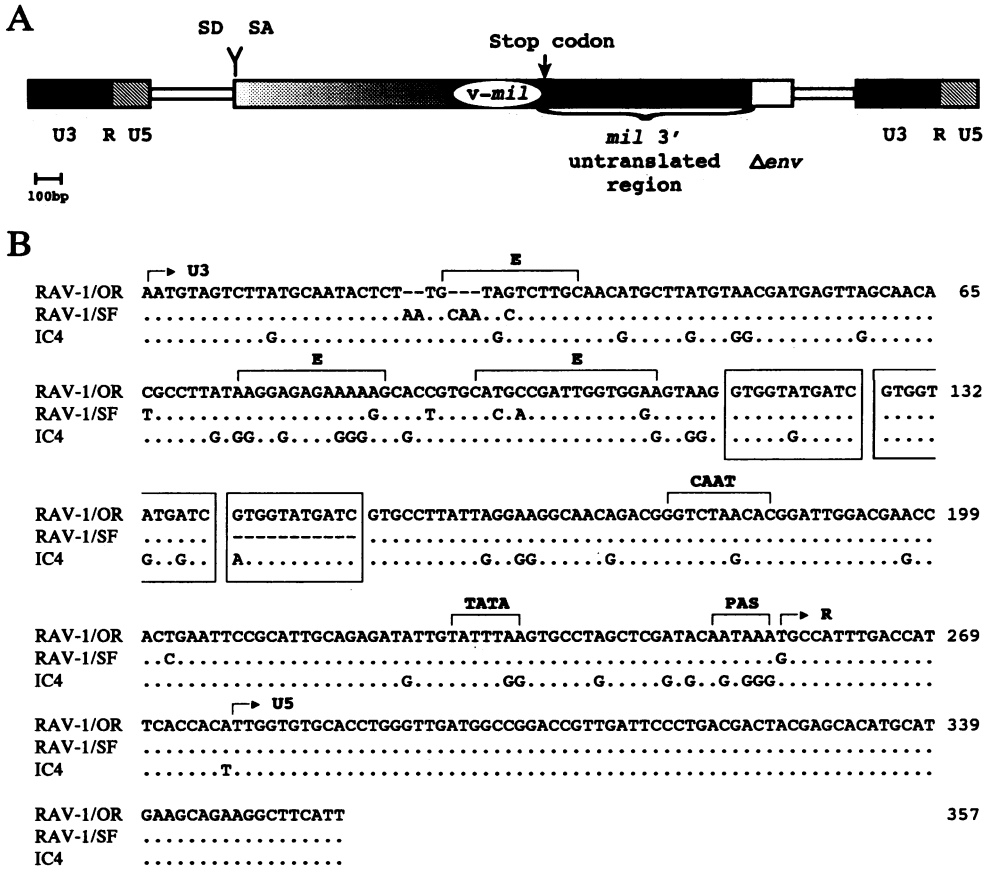

FIG. 2. (A) Genetic organization of IC4 provirus. SD, splice donor site of RAV-1 leader sequence; SA, splice acceptor site of exon 8 of the c-*mil* gene. The RAV-1-derived untranslated sequences are indicated by thin white boxes. (B) LTR sequences of RAV-1/OR, RAV-1/SF, and IC4 proviruses. The 5′ extremities of the U3, R, and U5 regions are indicated by arrows. Identical nucleotides are indicated by dots. TATA and CAAT boxes (13), enhancers (E) (8, 15, 39), and the PAS (13) are indicated. The sequence similar to that of the SV40 enhancer core is boxed. Bases are numbered in reference to the first nucleotide of U3 of RAV-1/OR.

cells leads to the generation of replication-defective retroviruses containing the catalytic domain of c-*mil*/c-*raf* or c-R*mil*/B-*raf* proto-oncogenes (11, 27, 28). In the course of one such experiment, we identified and molecularly cloned a novel c-*mil*-containing provirus, designated Institut Curie 4 (IC4) (Fig. 2A). The IC4 sequence is 3,026 nucleotides long. It contains 5′ and 3′ LTRs, the leader sequence of RAV-1, 1,110 nucleotides derived from the 3′ coding portion of c-*mil* including the kinase domain of the gene, and 761 nucleotides derived from the 3′ noncoding region of c-*mil*. It also contains the last 215 nucleotides of the *env* gene and the 3′ untranslated region of RAV-1. The 5′ viral sequences of IC4 were acquired through a splicing mechanism between the leader of RAV-1 and the 8th exon of c-*mil*, whereas its 3′ viral sequences were acquired by an illegitimate recombination mechanism involving sequence similarities between c-*mil* and RAV-1 genomes (data not shown). These results indicated that IC4 was generated by the same mechanisms which gave rise to all early forms of *mil*- and R*mil*-transducing retroviruses previously described (10–12).

The LTR of IC4 contains 357 nucleotides. We compared the nucleotide sequence of this LTR with those of our strain of RAV-1 used in oncogene transduction experiments (RAV-1/OR) and of pRAV-1/SF, a molecular clone of RAV-1/SF (a gift of J. M. Bishop), both sequenced in our laboratory (Fig. 2B). The R and U5 sequences of all three viruses are almost identical. In contrast, the U3 sequence of IC4 is highly mutated in comparison to that of the other viruses. Specifically, 37 of 77 (48%) A are converted to G. These mutations are dispersed throughout the U3 sequence and lead to major sequence changes in all viral regulatory elements: enhancers, promoter, and PAS.

Further sequence comparison of these LTRs revealed two deletions of two and three bases at positions 24 and 25 and 28 to 30, respectively, in the U3 sequences of RAV-1/OR and IC4 (Fig. 2B). We also found that the GTGGTATGATC sequence is triplicated between nucleotides 121 and 155 (Fig. 2B) in both RAV-1/OR and IC4. This sequence, which is only duplicated in RAV-1/SF, presents similarities with the core enhancer sequence of the simian virus 40 (SV40) 72-bp repeat and the murine sarcoma virus LTR (22). The homologous sequence in RSV (present as a single copy) has been previously proposed to act as an enhancer (23, 25). Interestingly, the same triplication occurs in the U3 sequence of ring-necked pheasant virus (RPV) (41). A correlation between RPV U3 sequence and its ability to induce lymphoid leukosis after an unusually short latent period has been reported (40). Moreover, RPV has been described to replicate at a higher titer in comparison to other avian leukemia viruses, which may be also due to more effective enhancer sequences (41). The presence of this triplication could, therefore, explain in part the high transducing efficiency of RAV-1/OR.

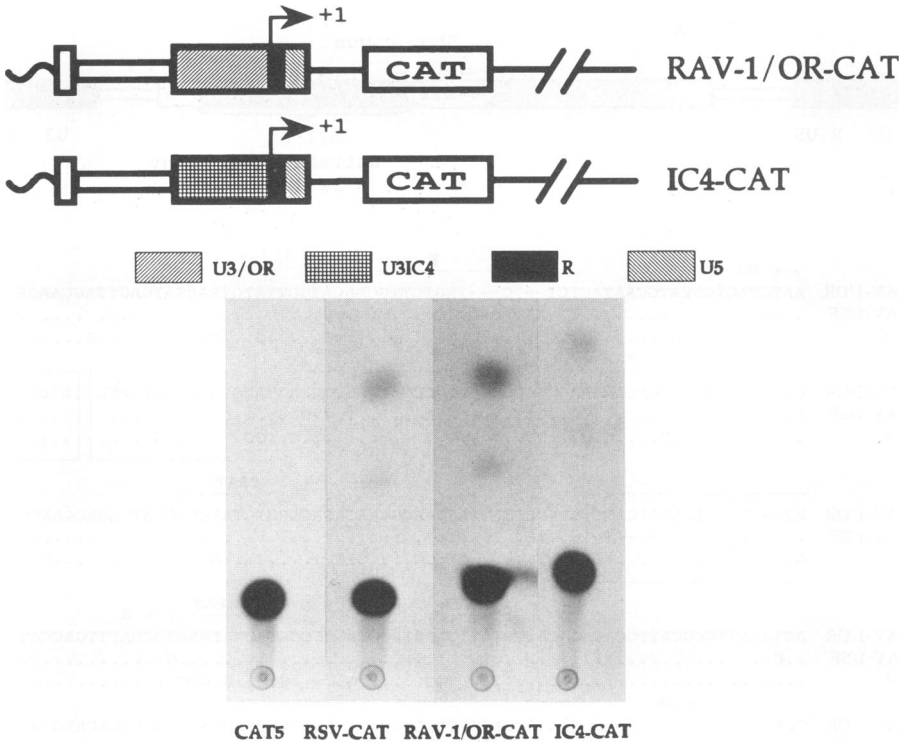

FIG. 3. Transcriptional activity of IC4 LTR sequences. Plasmids designed to analyze the ability of IC4 and RAV-1/OR LTR sequences to drive transcription are shown. The *Hin*dIII-*Nsi*I fragments of pLTRIC4 and pLTRRAV-1/OR plasmids were inserted into the promoterless pBLCAT5 vector. These fragments contain the whole U3 and R elements and the first 56 nucleotides of U5. The pBLCAT5 vector was used as a control. These plasmids were cotransfected with the pSVβgal into NR cultures. CAT activity in cell extracts was measured after adjustments were made according to β-galactosidase activity.

Because of conserved sequence similarities in U3 of IC4 and of RAV-1/OR, namely, positions of deletions and presence of sequence triplication, we believe that IC4 LTR was derived from RAV-1/OR LTR by mutation during replication. The mechanism(s) which could generate such a high rate of mutations is unknown. We considered the possibility that high retroviral mutability during reverse transcription could explain IC4 U3 genesis. Hot spots of mutations in avian retroviruses undergoing one cycle of replication have been described. However, essentially G to A changes have been observed (32).

An enzymatic activity converting A to I in double-stranded RNA (dsRNA) has been described for several cell types, notably mammalian and avian cells and amphibian eggs (48). Occurrence of such A to I modifications in retroviral transcripts would result in apparent A to G mutations after reverse transcription into dsDNA. The A to G changes present in the IC4 U3 sequence display most of the specificities shown to characterize such dsRNA modifying activity, namely, the involvement of up to 48% of A and the increased probability of modification when the A is preceded by an A or T (21). The alterations in IC4 U3 were, therefore, possibly induced by an analogous enzyme. Moreover, intramolecular hybrids can also undergo such alterations, provided they extend over 20 bp (30). According to models of secondary structures (9), retroviral transcripts are likely to be involved in extensive intramolecular duplexes and are thus potential substrates for the dsRNA-modifying enzyme. The reasons for limitation of the A to G modifications to the U3 sequence are unclear. One hypothesis is that it is only the remnants of larger alterations which were eliminated during replication.

Strikingly, analogous A to G mutations in isolates of other RNA viruses, notably, vesicular stomatitis virus (31), hepatitis delta virus (26), measles virus (1), and respiratory syncytial virus (36), have been described and were assigned to the enzymatic activity mentioned above. Our results suggest, for the first time, that the dsRNA-modifying enzyme described by Wagner et al. (47) also induces A to G mutations in retroviral genomes. It is noteworthy that such enzymatic activity could also contribute to the high variability which has been reported for retroviruses (20).

There are two possible explanations for the presence of mutated U3 sequences in IC4 virus. It may be that A to I changes occurred during IC4 replication. Alternatively, it is possible that the mutated sequences would have been acquired during recombination with a subpopulation of RAV-1 carrying a modified U3 region.

Functional properties of IC4 LTR. To analyze the ability of mutated U3 sequences to promote transcription, we inserted the *Hin*dIII-*Nsi*I fragment of pLTRIC4 into the promoterless CAT pBLCAT5 plasmid (43). A construct containing the corresponding sequences of RAV-1/OR was prepared. The RSV-CAT plasmid (14), which contains a similar extent of LTR sequences, was used as a positive control. We transfected plasmid DNAs into chicken NR cells and measured CAT activity 20 h after transfection (Fig. 3). Like RSV-CAT and, to a lesser extent, like RAV-1/OR-CAT, the IC4-CAT construct displayed significant CAT activity. We conclude that the IC4 LTR is efficient in driving transcription, despite its highly mutated U3 region.

To investigate the cleavage and polyadenylation properties

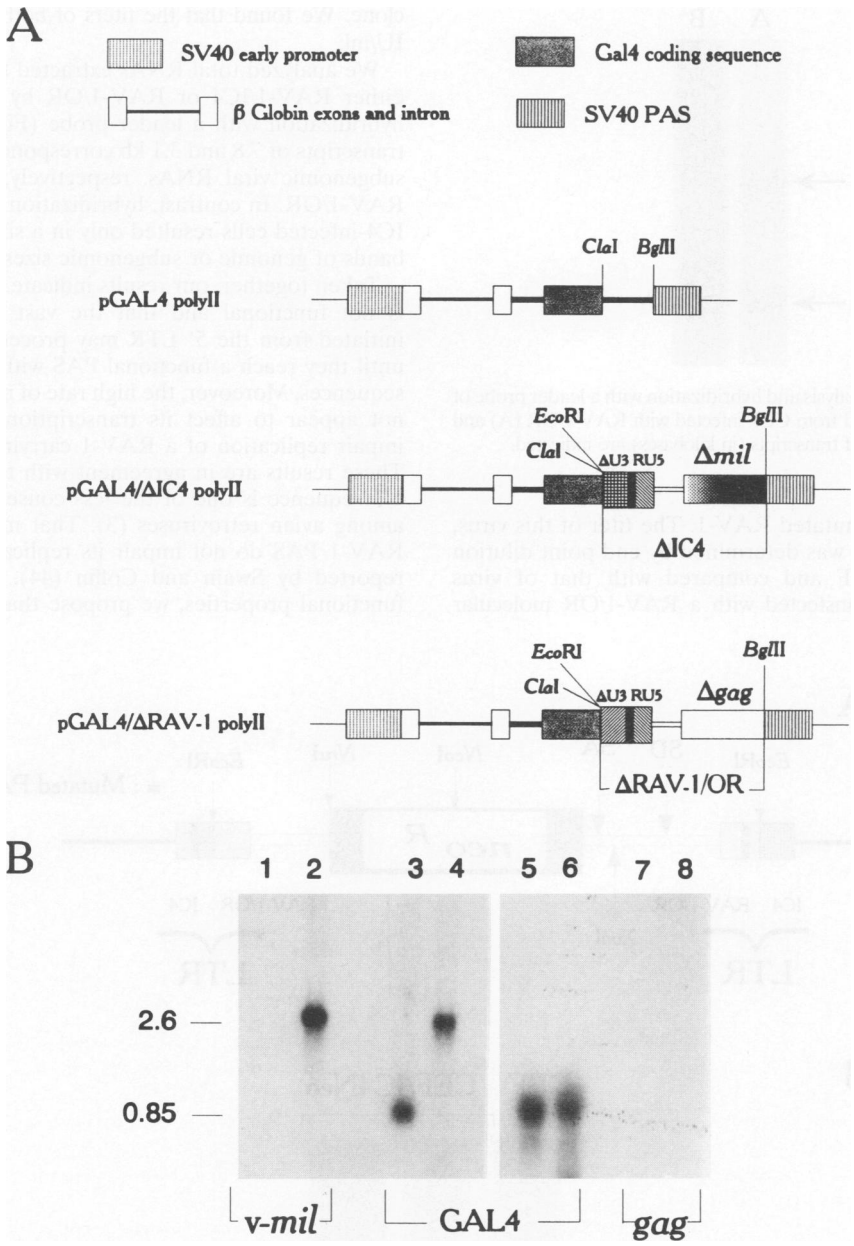

FIG. 4. Functional characterization of PAS of the IC4 LTR. (A) pGAL4polyII-derived expression vectors containing the PAS of IC4 (pGAL4/ΔIC4polyII) and the PAS of RAV-1/OR (pGAL4/ΔRAV-1polyII). (B) Northern blot analysis of RNAs extracted from COS-1 cells transiently transfected with pGAL4polyII (lanes 1, 3, 5, and 7), with pGAL4/ΔIC4polyII (lanes 2 and 4) and with pGAL4/ΔRAV-1polyII (lanes 6 and 8).

of pIC4 LTR, we prepared two expression plasmids derived from the pGAL4polyII vector (5, 50) (Fig. 4A). The first one, pGAL4ΔIC4polyII, which contains the PAS of IC4, was obtained by inserting the 1.8-kbp *Cla*I-*Bgl*II fragment of IC4 (Fig. 1). The second one, pGAL4ΔRAV-1polyII, which contains the RAV-1/OR PAS together with *gag* sequences, was obtained by inserting the 1.7-kbp *Cla*I-*Bgl*II fragment of pRAV-1/OR (Fig. 1). We transfected both plasmids into COS-1 cells and extracted total cellular RNA 48 h later. The contents of these RNAs were analyzed by Northern blotting and hybridization with GAL4, v-*mil*, and *gag* probes (Fig. 4B). Cells transfected with pGAL4ΔIC4polyII synthesized a readthrough RNA of 2.6 kb hybridizing to both GAL4 and v-*mil* probes (Fig. 4B, lanes 2 and 4), indicating that IC4 PAS is not functional and allows transcription to proceed to the SV40 PAS located downstream. In contrast, cells transfected with pGAL4ΔRAV-1polyII synthesized an RNA of 0.85 kb which hybridized only with the GAL4 probe (Fig. 4B, lanes 6 and 8).

We also studied the effects of IC4 LTR mutations on viral transcription and replication by preparing a RAV-1-derived recombinant virus carrying the mutated TATA box and PAS in the U3 region. Therefore, we inserted the 7.8-kbp *Eco*RI fragment obtained after partial digestion of pRAV-1/OR into the pLTRIC4 vector, and we transfected this plasmid DNA

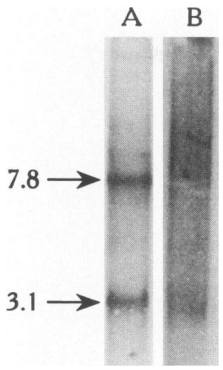

FIG. 5. Northern blot analysis and hybridization with a leader probe of 10 μg of total RNA extracted from CEF infected with RAV-1/OR (A) and with RAV-1/IC4 (B). Sizes of transcripts (in kilobases) are indicated.

into CEF to generate mutated RAV-1. The titer of this virus, designated RAV-1/IC4, was determined by end point dilution on newly infected CEF and compared with that of virus produced from CEF transfected with a RAV-1/OR molecular clone. We found that the titers of both viruses were about 10^6 IU/ml.

We analyzed total RNAs extracted from CEF infected with either RAV-1/IC4 or RAV-1/OR by Northern blotting and hybridization with a leader probe (Fig. 5). We detected two transcripts of 7.8 and 3.1 kb corresponding to the genomic and subgenomic viral RNAs, respectively, in CEF infected with RAV-1/OR. In contrast, hybridization of RNAs from RAV-1/IC4-infected cells resulted only in a smear without detectable bands of genomic or subgenomic sizes (Fig. 5).

Taken together, our results indicate that the PAS in IC4 U3 is not functional and that the vast majority of transcripts initiated from the 5′ LTR may proceed through the 3′ LTR until they reach a functional PAS within downstream cellular sequences. Moreover, the high rate of mutation in IC4 U3 does not appear to affect its transcriptional activity, nor does it impair replication of a RAV-1 carrying IC4 LTR sequences. These results are in agreement with the observation that the U3 sequence is one of the less conserved regulatory regions among avian retroviruses (3). That mutations limited to the RAV-1 PAS do not impair its replicative efficiency was also reported by Swain and Coffin (44). On the basis of their functional properties, we propose that such RAV-1 variants,

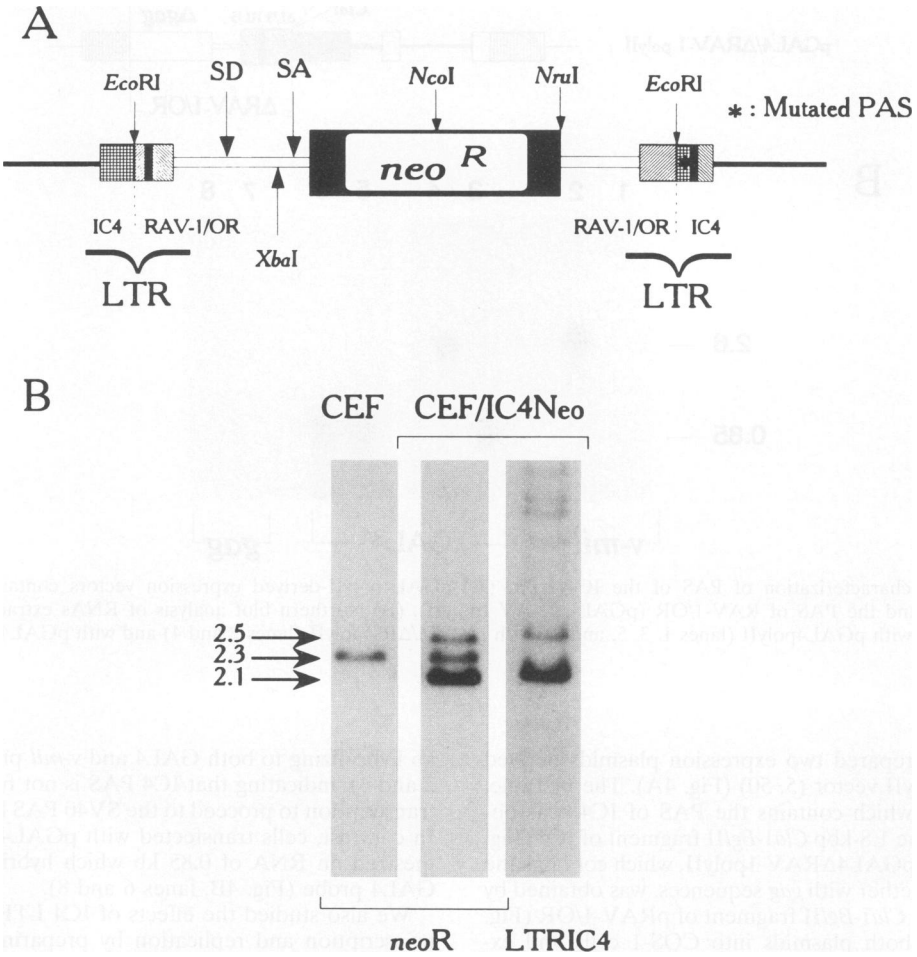

FIG. 6. (A) Genomic organization and restriction mapping of pIC4Neo vector. SD, splice donor site; SA, splice acceptor site. (B) *Eco*RI digestion, Southern blot analysis, and hybridization with Neo^r and LTR probes of genomic DNA extracted from noninfected CEF and from CEF infected with IC4Neo virus. The 2.3-kbp fragment revealed by the Neo^r probe corresponds to retroviral sequences contained in this probe located between the *Xba*I site and the first nucleotide of the Neo^r gene (2). Sizes of DNA fragments (in kilobase pairs) are indicated.

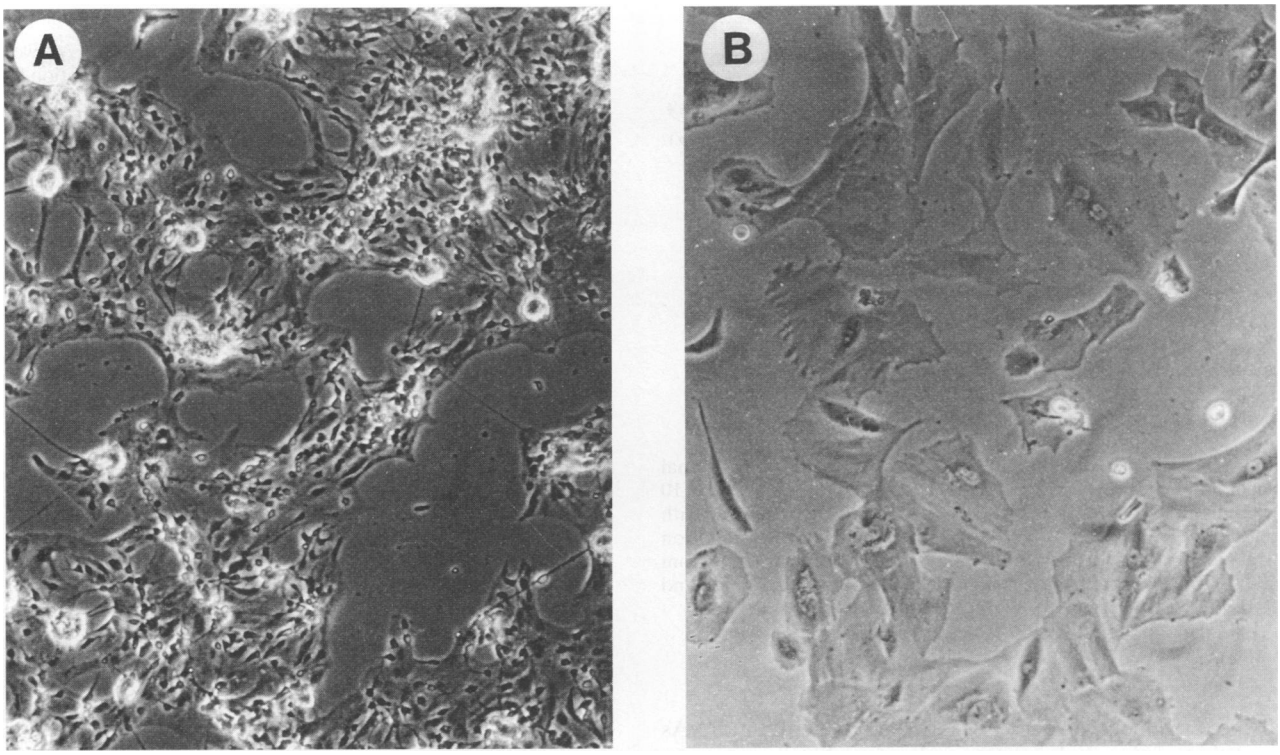

FIG. 7. Morphologies of noninfected quiescent NR cells (A) and of the proliferating 4NR-IC4Neo culture (B).

occurring naturally, could contribute to the activation of cellular genes by increasing the rate of readthrough transcription. This is in agreement with the results of Swain and Coffin, stating that occurrence of readthrough transcripts due to a RAV-1 carrying a mutated PAS strongly increases the transduction rate of sequences located downstream of the provirus (45).

A retroviral vector with IC4-derived LTR induces NR cell proliferation. Retroviral vectors carrying mutated PAS are potentially useful as insertion mutagens. Characterization of readthrough transcripts generated by such vectors should make the identification of cellular downstream sequences easier. Therefore, we constructed an IC4-based vector, designated pIC4Neo (Fig. 6A), and we used it as a nonreplicative virus to test its capacity to activate NR cell division. Enhancers and PAS of this plasmid are provided by IC4, whereas the promoter (TATA box) is provided by RAV-1/OR. This plasmid also contains the leader sequence of RAV-1 with its splice donor site, part of the *gag* gene, the neomycin resistance (Neo^r) gene with a splice acceptor site, and 3′ noncoding retroviral sequences. We introduced this plasmid DNA into a quail packaging cell line (7) and selected, after treatment with G418, a cell population that released replication-defective virus able to efficiently confer resistance to this antibiotic upon newly infected cells. The titer of IC4Neo virus, determined by testing the ability of 10-fold dilutions of packaging cell supernatant to transfer G418 resistance to infected CEF, was about 10^3 IU/ml. To determine the structure of virions generated by packaging cells, we analyzed DNA extracted from G418-resistant infected CEF by Southern blotting and hybridization with Neo^r and IC4 LTR probes (Fig. 6B). We detected the presence of two *Eco*RI DNA fragments of 2.5 and 2.1 kbp, consistent with the expected sizes generated from unspliced and spliced forms, respectively, of the vector. It is noteworthy that the expected U3 sequence of virions produced from packaging cells should be identical to that of the 3′ LTR of pIC4Neo (Fig. 6A). These results indicate that both unspliced and spliced transcripts of the vector are synthesized in packaging cells and generate two forms of infectious virions. However, on the basis of the relative amounts of both proviruses, the spliced RNA form appears to be either more efficiently transcribed or more efficiently packaged. The transcripts generated by the IC4Neo provirus were analyzed by Northern blotting of total RNA extracted from G418-resistant, infected CEF (data not shown). We detected several transcripts hybridizing with the Neo^r and the leader probes, all of which were longer than the viral genome. These results indicate that the IC4Neo provirus is able to generate only readthrough transcripts.

We infected 10 dishes of NR cells with IC4Neo virus and maintained the cells in selective medium. Seven weeks after infection, we observed the presence of one focus of proliferating cells in 3 of 10 infected dishes. They consisted of a small number of flat cells with an epithelium-like morphology, clearly different from that of cells expressing activated oncogenes (Fig. 7). Two cultures displayed growth capacity limited to 3 and 10 passages. The third one, designated 4NR-IC4Neo, proliferated for about 30 generations. We analyzed DNA from these cells by Southern blotting and hybridization with Neo^r and IC4 LTR probes (Fig. 8A). We detected a unique *Eco*RI DNA fragment of 2.1 kb, presumably generated by the spliced form of the vector. DNA digestion with *Xba*I revealed a unique fragment of 7 kbp which hybridized with both probes. These results suggested that these cells contain a single copy of IC4Neo provirus, expected to be expressed only as a chimeric RNA containing both retroviral and cellular sequences. To verify this possibility, we analyzed total RNA extracted from 4NR-IC4Neo cells by Northern blot and hybridization. We detected two transcripts of 4.6 and 3.2 kb containing leader and

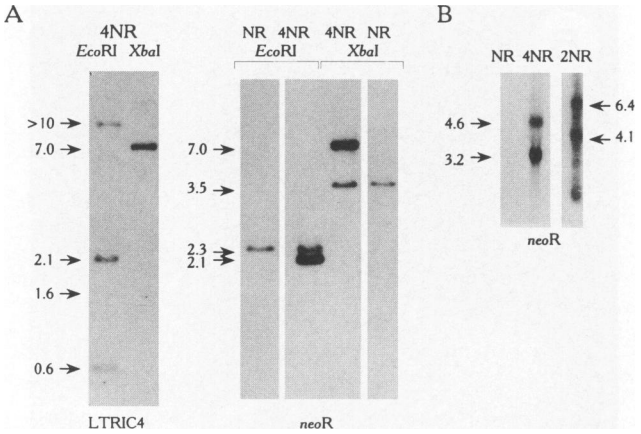

FIG. 8. (A) Southern blot analysis of DNA extracted from normal NR cells and from proliferating 4NR-IC4Neo cells. Genomic DNA (10 μg) was digested with *Eco*RI or with *Xba*I, blotted, and hybridized with Neo^r, and LTR probes. (B) Northern blot analysis and hybridization with a Neo^r-specific probe of 10 μg of total RNA extracted from normal NR cells and from proliferating 4NR-IC4Neo (4NR) and 2NR-IC4Neo (2NR) cells.

Neo^r gene sequences (Fig. 8B). The sizes of these RNAs exceed that of the viral genome, indicating that they contain cellular sequences downstream of the vector. To obtain information on these sequences, we analyzed total cellular RNA by the reverse transcription-PCR technique. We reverse transcribed the cellular RNA by using an oligo(dT) primer and then amplified cDNAs by using a Neo^r-specific oligonucleotide, ATCTCATGCTGGAGTTCTTC, as a 5′ primer and a poly(A)-specific oligonucleotide, GAGCTCGAGTCGCGAT GC17T, as a 3′ primer. An amplification product of 1.5 kbp hybridizing with an IC4 U3-specific oligonucleotide was cloned into the *Sma*I site of pUC18 vector. Sequencing data indicated that this DNA contains 770 nucleotides from the 3′ portion of IC4Neo virus, including the 3′ LTR as well as 710 bp of cellular origin. These cellular sequences include a PAS (AATAAA) located 20 bp upstream of an 18-A tract. A probe containing these 710 bp hybridized with both the 4.6- and 3.2-kb transcripts in 4NR-IC4Neo cells (data not shown), suggesting that these mRNAs are generated by a readthrough transcription initiated at the 5′ LTR of the provirus and then by either an alternative polyadenylation or by an alternative splicing of the cellular sequences contained in the primary transcript. Another proliferating culture independently infected with IC4Neo virus, 2NR-IC4Neo, contained transcripts which hybridized with Neo^r and leader probes and which were longer (4.1 and 6.4 kb) than the viral genome (Fig. 8B). Interestingly, these transcripts also hybridized with the 710-bp probe (data not shown). This strongly suggests that in both cultures cell proliferation is correlated with vector integration within the same locus.

In conclusion, we showed that infection of NR cells with IC4Neo virus may result in sustained proliferation, presumably as a consequence of insertional mutagenesis. This mitogenic activity is correlated with the generation of readthrough transcripts containing cellular sequences downstream of the 3′ LTR. Further characterization of these sequences should help in understanding the mechanisms which activate NR cell division.

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